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Age-Dependent Role for CCR5 in Antiviral Host Defense against Herpes Simplex Virus Type 2

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Elimination of viral infections is dependent on rapid recruitment and activation of leukocytes with antiviral activities to infected areas. Chemokines constitute a class of cytokines that have regulatory effects on leukocyte migration and activity. In this study we have studied the role of CC chemokine receptor 1 (CCR1) and CCR5 in host defense during a generalized herpes simplex virus type 2 (HSV-2) infection. Whereas both 4- and 8-week-old CCR1$^{-/-}$ mice resembled wild-type mice (C57BL/6) with respect to defense against the infection, significantly higher virus titers were seen in the livers and brains of 4-week-old CCR5$^{-/-}$ mice. At the age of 8 weeks, CCR5$^{-/-}$ were indistinguishable from wild-type mice and cleared the infection from liver and spleen. Although 4-week-old CCR5$^{-/-}$ mice were able to recruit natural killer (NK) cells to the site of infection, these cells had reduced cytotoxic activity compared to NK cells from wild-type mice. This was not due to lower production of alpha/beta interferon or interleukin-12, two well-described activators of cytotoxic activity in NK cells. We also noted that the spleens of young CCR5$^{-/-}$ mice did not increase in size during infection as did the spleens of wild-type and CCR1$^{-/-}$ mice. This observation was accompanied by impaired proliferation of CCR5$^{-/-}$ splenocytes (SCs) ex vivo. Moreover, migration of CD8^{+} T cells to the liver in response to infection was impaired in CCR5$^{-/-}$ mice, and adoptive transfer of SCs from CCR5$^{-/-}$ mice infected for 6 days into newly infected wild-type mice did not improve antiviral activity in the liver, in contrast to what was seen in mice receiving immune SCs from wild-type mice. Altogether, this study shows that CCR5 plays an age-dependent role in host defense against HSV-2 by supporting both the innate and adaptive immune response.

Establishment of an efficient immune response to infections is dependent on rapid recruitment of cells of the immune system to the infected area. In response to infections, the organism mounts an inflammatory host response aiming at eliminating the infectious agent. The best-described effector cell populations in defense against viral infections are natural killer (NK) cells and cytotoxic T lymphocytes (CTLs; CD8^{+} T cells). NK cells are lymphoid cells that develop in a bone marrow-dependent but thymus-independent manner (2). They can be activated to produce cytokines and exert perforin-dependent lysis of virus-infected cells by targeting cells expressing low levels of major histocompatibility complex (MHC) class I on the cell surface. NK cells are recruited to sites of viral infections at the early stages of infection (28), where cytotoxic activity is potently stimulated notably by alpha/beta interferon (IFN-α/β) (23). At later time points during infection, the adaptive immune system takes over, and CD8^{+} T cells specifically kill cells displaying the appropriate viral peptides in the context of MHC I (35). Maturation of antigen-specific T cells is initiated by antigen-loaded dendritic cells (DCs) and other antigen-presenting cells that migrate to secondary lymphoid organs and present antigens to T cells in the T-cell zones. Cells that receive signals through the T-cell receptor, as well as through costimulatory pathways, rapidly start to expand. T-cell proliferation is controlled both through cell-cell interactions and cytokine secretion. Once the T cells become activated, receptors are expressed on the cell surface, which allows the cells to migrate to infected areas of the body to kill virus-infected cells.

Regulation of leukocyte migration to lymphoid organs and inflamed areas is a complex process in which chemokines play pivotal roles (18). Chemokines constitute a large family of secreted peptides, which are produced by tissue cells or leukocytes in both constitutive and inducible manners (19). Chemokines are categorized into the subfamilies CXC (α), CC (β), C (γ) and CX_3C (δ), according to the positions of conserved amino-terminal cysteines (16, 19). Chemokine receptors are seven-transmembrane-spanning, G-protein-coupled receptors. They are classified based on the class of chemokines they bind, e.g., CCRs bind CC chemokines (CCLs), and CXCRs bind CXC chemokines (CXCLs). Among chemokines, there is a great degree of redundancy; i.e., many receptors bind several chemokines, and many chemokines use more than one receptor. Chemokine receptors are expressed primarily on leukocytes but also on other cells, such as endothelial cells (16).

With regard to the role of chemokines in defense and disease during viral infections, some information is available, mainly from studies in murine models (20). For instance, defense against intracerebral infection by mouse hepatitis virus (MHV) is impaired if either of the two CXCR3 ligands CXCL9 or CXCL10 is inactivated, which was suggested to be due to important roles of these chemokines in T-cell recruitment to the central nervous system (8, 15). Mice lacking CCR1 or the ligand CCL3 display impaired ability to clear infection by a paramyxovirus in the lower respiratory tract (7), which was associated with higher mortality and reduced inflammatory response (7). Also, clearance of systemic infection with the herpesvirus cytomegalovirus (CMV) was delayed in CCL3$^{-/-}$ mice, which correlated with decreased infiltration of NK cells

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to the liver (27). For some other virus infections, CCR1 or CCL3 do not contribute to host defense but are involved in the pathogenesis of the infections (3, 6, 32).

CCR5 is coreceptor for human immunodeficiency virus (HIV), and this molecule is thus a potential target for anti-HIV drugs (33). There is therefore a need for evaluation of the role of CCR5 in the immune response against infections. Although most work done in animal models has concentrated on adult mice (1, 5, 10, 14, 22, 36), little information is available on the function of CCR5 during infection in young mice, which have a less developed cellular immune response and hence are more prone to infections in general (21, 25). Host defense against the viruses lymphocytic choriomeningitis virus, influenza A virus or MHV, or the intracellular bacterium *Listeria monocytogenes* has been found not to be hampered in adult CCR5-deficient mice (5, 10, 22, 36), whereas lack of this receptor did impair defense against the encapsulated yeast *Cryptococcus neoformans* and the parasite *Toxoplasma gondii* (14, 24). In humans, natural CCR5 mutations are well described (13). By far the one most studied is the CCR5 Δ32 frameshift mutation, which is relatively abundant (17). Individuals homozygous for the CCR5 Δ32 mutation are fully immunocompetent with respect to defense against many infections but have been shown to display enhanced susceptibility to acute and chronic hepatitis C virus infection (34), thus suggesting a role for this receptor in both innate and adaptive immunity against some virus infections.

We have examined here the role of CCR1 and CCR5 in defense against a systemic herpes simplex virus type 2 (HSV-2) infection in 4- versus 8-week-old mice and found that CCR5 modulates both the innate and adaptive immune system in young animals and hence is essential for optimal antiviral defense early in life.

MATERIALS AND METHODS

Reagents. The growth media used were Eagle minimal essential medium (MEM), Hanks balanced salt solution (Gibco), and RPMI 1640. MEM and RPMI 1640 (both BioWhittaker) were supplemented with antibiotics (penicillin [200 IU/ml] and streptomycin [200 μg/ml]) and lipopolysaccharide-free fetal calf serum (FCS; BioWhittaker) at the indicated concentrations. MEM was also supplemented with 2% glutamine, 0.5% nystatin, 10% $NaHCO_3$, and 0.1% garamycin. RPMI 1640 was also supplemented with 1% glutamine and 1% HEPES (BioWhittaker). Heparin was obtained from Leo Pharma. TRIzol was from Invitrogen. Recombinant chemokines, capture and detection Abs used in enzyme-linked immunosorbent assays (ELISAs) were from R&D Systems. Both streptavidin-horseradish peroxidase and TMB substrate were purchased from R&D Systems. Bovine serum albumin (BSA) was from Sigma-Aldrich. Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies used for flow cytometry were from BD Pharmingen. Collagenase IV and metrizamide were from Sigma-Aldrich, and DNase I was from Roche.

Mice and virus. The mice used in the present study were 4- and 8-week-old female C57BL/6, $CCR1^{-/-}$ and $CCR5^{-/-}$ mice. The animals were bred at Taconic M&B (Ry, Denmark). The $CCR1^{-/-}$ and $CCR5^{-/-}$ mice were generated as described elsewhere (9, 14). The virus used was the MS strain of HSV-2. The mice were infected intraperitoneally (i.p.) on day 0 with 10^6 PFU of virus suspended in 100 μl of phosphate-buffered saline (PBS). The animals were sacrificed on the indicated days postinfection (p.i.), and peritoneal cells (PCs) were harvested by lavage of the peritoneal cavity with cold PBS (pH 7.4) supplemented with 5% FCS and 0.4% heparin. Samples of spleen, liver, and brain were kept in RPMI 1640 or MEM on ice for further treatment or frozen at the time of harvest.

Virus plaque assay. Samples of liver, spleen, and brain were weighed, thawed, and homogenized three times for 5 s each time in MEM supplemented with 5% FCS just before use in the plaque assay. After homogenization the organ suspensions were pelleted by centrifugation at 1,620 × *g* for 30 min, and the supernatants were used for plaque assay as well as ELISAs (see below). Plaque assays were done on Vero cells seeded in MEM supplemented with 5% FCS at a density of 1.2×10^6 in 5-cm diameter plates and left overnight to settle. The cells were infected by incubation for 1 h at 37°C with 100 μl of serial dilutions of the organ suspension and 400 μl of medium, during which the tissue culture plates were rocked every 15 min to ensure even distribution of the virus. Subsequently, the organ suspensions were removed, and 8 ml of MEM was added to the plates. The medium was supplemented with 2.5 to 5% FCS, depending on how confluent the cells were at the time of incubation and with 0.2% human immunoglobulin. The cells were incubated at 37°C for 2 days and stained with 0.03% methylene blue, and the plaques were counted.

ELISA. Murine CCL3, CCL5, interleukin-12 (IL-12) p40, and IFN-γ were detected by ELISA. Maxisorp plates were coated overnight at room temperature with 100 μl of antibody (anti-CCL3, 4 μg/ml [R&D Systems]; anti-CCL5, 2 μg/ml [R&D Systems]; or anti-IL-12, 8 μg/ml [BD Pharmingen]) in coating buffer (Na_2CO_3, 15 mM; $NaHCO_3$, 35 mM; NaN_3, 0.02% [pH 9.6]). After blocking for at least 1 h at room temperature with 300 μl of 1% BSA in blocking buffer (PBS with 5% sucrose 0.05% NaN_3 [pH 7.4]), successive culture supernatants or recombinant murine CCL3, CCL5 (both R&D Systems), or IL-12 p40 (BD Pharmingen) were added to the wells (100 μl each) and incubated overnight at 4°C. Subsequently, wells were incubated at room temperature for 2 h with 100 μl biotinylated, anti-murine detection antibody (CCL3, 50 ng/ml [R&D Systems]; CCL5, 100 ng/ml [R&D Systems]; IL-12, 1 μg/ml [BD Pharmingen]) in a 0.1% suspension of BSA in TBS (Trizma, 20 mM; NaCl, 150 mM; 0.05% Tween 20 [pH 7.3]). Streptavidin-horseradish peroxidase (R&D Systems) diluted in 1:200 in Tris-buffered saline with 0.1% BSA was added, followed by incubation for 20 min. As a substrate 100 μl of the TMB substrate was added, and plates were incubated in the dark for an appropriate amount of time. The color reaction was stopped with 50 μl of 5% H_2SO_4, and the absorbance was measured at 450 nm with 570 nm as reference. Between each step the plates were washed three times with PBS–0.05% Tween 20 (pH 7.4). For detection of IFN-γ we used the monoclonal antibody Duoset (R&D Systems), and the protocol recommended by the manufacturer was followed. The detection limits of the ELISA were as follows: 31.25 pg/ml (CCL3) and 15.63 pg/ml (CCL5, IL-12 p40, and IFN-γ).

IFN-α/β bioassay. IFN-α/β bioactivity was measured by using a L929-cell-based bioassay. L929 cells (2×10^4 cells/well in 100 μl) in MEM with 5% FCS were incubated overnight at 37°C in successive twofold dilutions of samples or murine IFN-α/β as a standard. Subsequently, vesicular stomatitis virus (VSV/V10) was added to the wells, and the cells were incubated for 2 to 3 days. The dilution mediating 50% protection was defined as 1 U of IFN-α/β/ml.

RT-PCR. Total RNA was extracted from PCs and organ homogenates with TRIzol according to the recommendations of the manufacturer. Briefly, cells were lysed in TRIzol, and chloroform was added, followed by phase separation by centrifugation. RNA was precipitated with isopropanol and pelleted by centrifugation. Pellets were washed with 70% ethanol and redissolved in RNase-free water. Then, 2 to 4 μg of RNA was subjected to reverse transcription (RT) with oligo(dT) as primer and Expand Reverse Transcriptase (both from Roche). The cDNA was amplified by PCR with the following primers: CCL3, 5′-GAA GAG TCC CTC GAT GTG GCT A-3′ (sense) and 5′-CCC TTT TCT GTT CTG CTG ACA AG-3′ (antisense); CCL5, 5′-ATA TGG CTC GGA CAC CAC TC-3′ (sense) and 5′-GAT GCC GAT TTT CCC AGG AC-3′ (antisense); and β-actin, 5′-CCC ACT CCT AAG AGG AGG ATG-3′ (sense) and 5′-AGG GAG ACC AAA GCC TTC AT-3′ (antisense). The products spanned 561 bp (CCL3), 330 bp (CCL5), and 215 bp (β-actin).

Isolation of splenocytes (SCs). Spleens were harvested and kept in 1 ml of RPMI 1640 supplemented with 10% FCS. Spleens were homogenized manually five times with a glass homogenizer in 1 ml of RPMI supplemented with FCS. The suspensions were filtered through gauze, which was washed with 10 ml of medium. The suspensions were pelleted by centrifugation at 180 × *g* for 10 min. The erythrocytes were lysed in NaCl 0.2% for 30 s, followed by the addition of an equal volume of NaCl 1.6% supplemented with 10% FCS. The leukocytes were pelleted by centrifugation at 115 × *g* for 12 min and resuspended in RPMI 1640 supplemented with 10% FCS.

Isolation of IHLs. Livers were isolated and washed repeatedly in RPMI 1640 supplemented with 5% FCS, followed by manual homogenization with a glass homogenizer. The homogenate was incubated in RPMI 1640 with 0.02% (wt/vol) collagenase IV and 0.002% (wt/vol) DNase I at 37°C for 40 min. Cells were precipitated by centrifugation at 720 × *g* for 10 min, resuspended in RPMI 1640, underlaid with 26% (wt/vol) metrazamide in PBS, and centrifuged at 1,500 × *g* for 20 min. Intrahepatic leukocytes (IHLs) were isolated in the interface, and erythrocytes were lysed in 0.2% NaCl for 30 s, followed by the addition of an equal volume of 1.6% NaCl. The leukocytes were pelleted by centrifugation for 10 min at 180 × *g*.

Purification of T cells. To isolate T cells from the spleen, we used the Pan T Cell Isolation kit (Miltenyi), following the instructions of the manufacturer, and separated the cells with an AutoMACS cell sorter. Cell purification with this kit yields untouched T cells depleted of B cells, NK cells, DCs, macrophages, granulocytes, and erythroid cells.

NK cell cytotoxicity assay. YAC-1 cells were loaded with BATDA [bis(acetoxymethyl)2,2′:6′,2″-terpyridine-6,6″-dicarboxylate] by incubating the cells for 20 min at 37°C in RPMI supplemented with 10 μM BATDA. The cells were washed four times and seeded in 96-well plates with 5×10^3 cells per well. PCs were added to the target cells in the indicated ratios to reach a final volume of 200 μl per well, and effector and target cells were brought into contact by centrifugation for 5 min at 50 × g. The cells were incubated for 2 h at 37°C, after which the cells were resuspended and centrifuged to allow even distribution of the released BATDA. From each well 25 μl was mixed with 200 μl of a 50 μM Eu^{3+} solution. Fluorescence was measured, and the percent cell lysis was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Measurement of ex vivo production of IFN-γ by splenic T cells. To assay production of IFN-γ by T cells, spleens were harvested on day 6 p.i and kept in 1 ml of cold RPMI 1640 supplemented with 5% FCS. Splenic T cells were isolated as described above and set in culture in 24-well culture plates (2×10^6 cells/well in 1 ml) with irradiated syngeneic SCs as antigen-presenting cells. The cells were either left untreated or received 3×10^7 PFU of heat-inactivated HSV-2. Supernatants were collected after 72 h of culturing and IFN-γ was measured by ELISA as described above.

Measurement of SC proliferation. To assay proliferation of SCs, spleens were harvested on day 5 p.i., and SCs were isolated as described above. The cells were set in culture in 96-well plates in a CO_2 humidified atmosphere. Proliferation was measured by using the EZ4U kit (Biomedica) according to the instructions of the manufacturer. Briefly, the substrate was dissolved and 20 μl was added per well to reach a final volume of 220 μl/well. After 4 h, absorbance was measured at 450 nm, with 620 nm as a reference.

Flow cytometry. PCs were harvested, counted, and resuspended in RPMI 1640 supplemented with 10% FCS to a concentration of 10^7 cells/ml, The cells were incubated in a 96-well plate on ice in the dark for 40 min with FITC-conjugated anti-mouse monoclonal anti-NK1.1 antibody at a concentration of 16.22 μg/ml. SCs and IHLs were isolated as described above. The leukocytes were incubated in a 96-well plate on ice in the dark for 40 min with FITC-conjugated anti-mouse monoclonal anti-CD3a antibody (16.22 μg/ml), PE-conjugated rat anti-mouse monoclonal anti-CD8a and CD4 antibodies (6.5 μg/ml), or combinations of the FITC- and PE-conjugated antibodies at the concentrations mentioned above. We used FITC-conjugated monoclonal mouse immunoglobulin G1 (16.22 μg/ml) and PE-conjugated monoclonal mouse immunoglobulin G2a (6.5 μg/ml) as isotype control antibodies.

The labeled cells were fixed by using 1% paraformaldehyde diluted in PBS and kept at 4°C until they were analyzed. Acquisition and analysis were performed with a flow cytometer (Coulter FS500). The data were stored in list mode files. A total of 20,000 cells were analyzed in each experiment by using a single laser system with a wavelength of 488 nm. Compensation was determined before the acquisition of data.

Adoptive transfer. Mouse donors (C57BL/6 and $CCR5^{-/-}$ mice) were infected i.p. with 10^6 PFU of HSV-2 prior to isolation of donor cell populations. For isolation of NK cell activity, PCs were harvested 24 h p.i. and cultured for 2 h in RPMI supplemented with 5% FCS before isolation of nonconfluent cells, which contained >95% of the cytotoxic activity. The cells (4×10^6 per mice) were injected i.p. into $CCR5^{-/-}$ mice infected with 10^6 PFU of HSV-2 2 h previously. The nonadherent cells contained between 10 and 400 virus particles per 4×10^6 cells as determined by plaque assay, with no differences between wild-type (WT) and $CCR5^{-/-}$ mice. For isolation of SCs, mice were sacrificed 6 days p.i., and cells were isolated as described above. The SCs were resuspended in Hanks balanced salt solution, and the desired number of viable cells were inoculated slowly intravenously into 4-week-old female C57BL/6 recipient mouse, which had been infected 6 h previously i.p. with 10^6 PFU of HSV-2 suspended in 100 μl of PBS. The SCs contained between 0 and 10 virus particles per 5×10^7 cells as determined by plaque assay, with no differences between WT and $CCR5^{-/-}$ mice. At 2 days after infection, livers were harvested and the viral load was determined by plaque assay.

Statistical analysis. The data are presented as means ± the standard error of the mean (SEM). The statistical significance was estimated with the Student *t* test for unpaired observations or Wilcoxon rank sum test. *P* values of <0.05 were considered significant.

RESULTS

Characterization of HSV-2 infection in 4- and 8-week-old C57BL/6, $CCR5^{-/-}$, and $CCR1^{-/-}$ mice. To examine how lack of CCR1 or CCR5 affected defense against HSV-2 infection, we infected age- and sex-matched C57BL/6, $CCR1^{-/-}$, and $CCR5^{-/-}$ mice i.p. with 10^6 PFU of HSV-2. Livers, spleens, and brains were harvested on days 1, 4, and 6 p.i., and virus titers were determined by plaque assay.

In the 4-week-old mice a generalized infection developed (Fig. 1A to C and G to I). In the spleen, virus was detected from day 1 p.i., and virus titers remained high through day 4, after which the amount of virus declined (Fig. 1B and H). No differences between viral load in the spleen of C57BL/6 and any of the knockout mouse strains were found. In the brain, virus was detected on day 6 in all mice strains (Fig. 1C and I). However, significantly higher virus titers were observed in CCR5-deficient animals on day 6 compared to WT C57BL/6 mice (Fig. 1I) ($P = 0.029$). In the livers of 4-week-old C57BL/6 mice, the virus was detectable from day 1 and peak virus titers were reached on day 4, followed by a pronounced decline in virus titer between days 4 and 6 (Fig. 1A and G). No significant differences between WT and $CCR1^{-/-}$ mice were observed (Fig. 1A). For CCR5-deficient mice, however, significantly higher virus titers were found on day 4 ($P = 0.014$) and day 6 ($P = 0.007$) compared to WT littermates (Fig. 1G). The strong decline between day 4 and day 6 seen in the WT and CCR1 mice was not found in the CCR5 mice, in which the virus titer remained high throughout the course of infection.

In the 8-week-old mice (Fig. 1D to F and J to L) we detected low levels of virus in the liver on days 1 and 4 p.i. (Fig. 1D and F). On day 6 p.i. the infection had been cleared, which is consistent with earlier findings (21). In the brain we detected significantly less virus than what was seen in 4-week-old mice at the same time p.i. (Fig. 1F and L). In the spleen virus was detected on days 1 and 4 p.i., and the infection was cleared from this organ by day 6 (Fig. 1E and K). No differences in the ability to clear the infection between the groups of 8-week-old mice were observed. These results show that CCR5, but not CCR1, is important for clearance of a generalized HSV-2 infection in young but not adult mice.

Expression of CCL3 and CCL5 during HSV-2 infection. To investigate the expression of ligands for CCR5 in vivo, 4-week-old C57BL/6 were infected i.p. with 10^6 PFU of HSV-2, and livers, spleens, and PCs were harvested on days 1, 4, and 6. RNA and soluble fractions of organ homogenates were prepared and used for RT-PCR and ELISA, respectively. PCs were either lysed for RNA purification immediately after the mice were sacrificed or cultured for 24 h for measurement of ex vivo chemokine production. As shown in Fig. 2A to C, HSV-2 infection triggered expression of CCL3 and CCL5 in the peritoneal cavity, with clear induction observed on day 1 p.i. The levels remained elevated until day 6 p.i. for CCL5 but not for CCL3, whose mRNA levels decreased significantly between days 1 and 4 p.i. At the protein level, a clear induction of both CCL3 and CCL5 was observed on day 1 p.i., after which a slight decrease was observed on days 4 and 6. In the liver, we observed elevated levels of both CCL3 and CCL5 proteins on day 1, which subsequently decreased modestly (Fig. 2D to E). The mRNA for CCL3 was found to be induced on day 1 and to

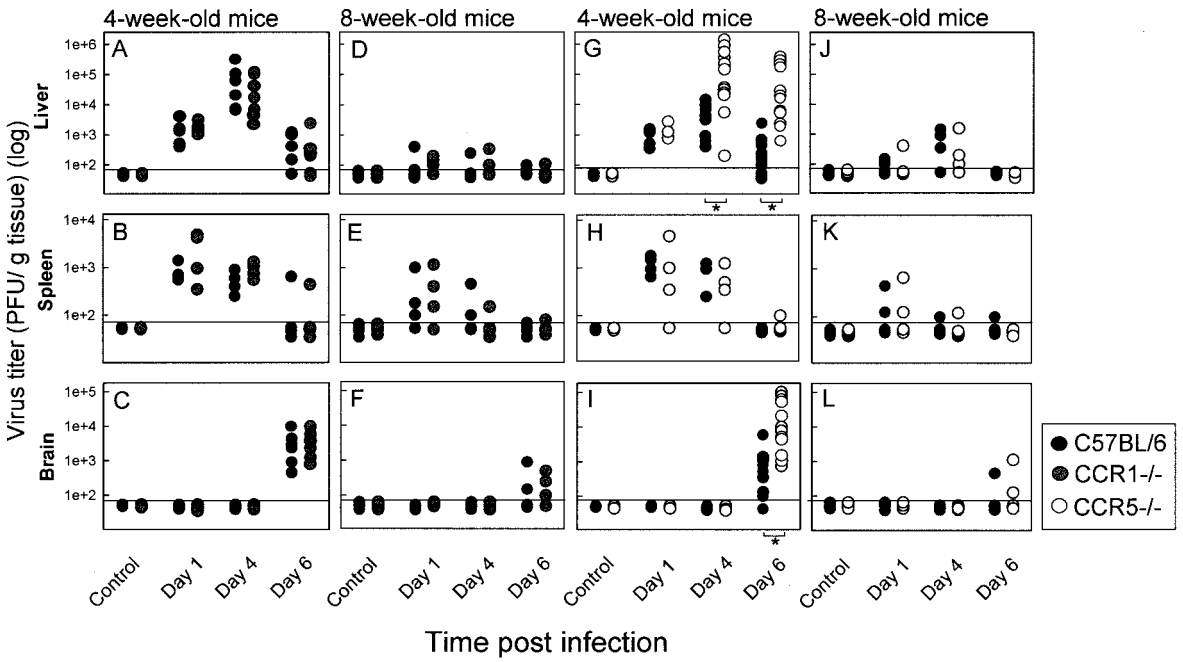

FIG. 1. Virus titers during a generalized HSV-2 infection in mice lacking CCR1 and CCR5. Four- and 8-week-old C57BL/6, CCR1$^{-/-}$, and CCR5$^{-/-}$ mice were infected i.p. with 10^6 PFU of HSV-2. At the indicated time points p.i. livers, spleens, and brains were harvested and HSV-2 titers were determined by plaque assay. The results are shown as dots represent individual mice. Statistically significant differences ($P < 0.05$) are marked by asterisks ($n = 4$ to 12).

remain high through the 6 days of the experiment (Fig. 2F), whereas for CCL5 the infection did not seem to affect mRNA levels. In the spleen, a clear induction of CCL3 protein and mRNA was observed on day 1 p.i., which decreased to basal levels on day 4 (Fig. 2G to I). CCL5 mRNA and protein was constitutively present and appeared not to be affected by the HSV-2 infection. Thus, CCL3 and CCL5, two ligands for CCR1 and CCR5, are induced during a generalized HSV-2 infection with distinct expression patterns in different organs.

NK cell recruitment and activity in the peritoneal cavity in the response to HSV-2 infection. Since the infection was initialized by i.p. inoculation, we wanted to characterize the early recruitment of antiviral effector cells to the peritoneal cavity. For this purpose PCs were harvested and counted on days 1, 4, and 6 p.i., and NK cells were identified by flow cytometry using an antibody to NK1.1. which is expressed predominantly, but not exclusively (30), on NK cells. HSV-2 infection resulted in a rapid and significant increase in the total number of PCs in both C57BL/6 and CCR1- and CCR5-deficient mice (Fig. 3A). No differences between the groups were found. When looking for NK cells in the peritoneal cavity, we observed a highly significant increase in the number of NK cells on day 1 p.i. in C57BL/6 ($P = 2.24 \times 10^{-7}$) and CCR1- ($P = 6.92 \times 10^{-6}$) and CCR5-deficient mice ($P = 0.0021$) (Fig. 3B to E). The CCR1$^{-/-}$ mice responded on day 1 with an even higher number of NK cells, compared to WT mice, whereas CCR5$^{-/-}$ mice were statistically indistinguishable from WT mice at all times examined. These results suggest that recruitment of NK cells to an area of infection is not inhibited in young CCR1- and CCR5-deficient mice.

When examining the ability of NK cells from the different mice to exert cytotoxic activity, we found that PCs from C57BL/6 mice and CCR1-deficient mice were indistinguishable with respect to cytotoxic activity toward killing of YAC-1 cells (Fig. 3F). However, PCs from infected CCR5-deficient mice displayed significantly impaired NK cell activity compared to PCs from WT mice despite unaltered recruitment of NK cells to the peritoneal cavity (Fig. 3G).

IL-12 and IFN-α/β are two well-described activators of NK cell activity (2, 31). Therefore, reduced production of any of these cytokines in response to infection could potentially explain the impaired cytotoxic activity. To test this, PCs were harvested from C57BL/6 and CCR5$^{-/-}$ mice and infected with HSV-2 for 24 h, and supernatants were examined for IL-12 and IFN-α/β. As seen in Fig. 4, HSV-2 infection triggered expression of both IL-12 and IFN-α/β, and no differences were observed between cells from C57BL/6 and CCR5$^{-/-}$ mice.

To examine whether activation of NK cells in the peritoneal cavity in response to HSV-2 infection had any impact on control of the virus in the liver and whether this might contribute to the difference in virus titers in the liver observed between C57BL/6 and CCR5$^{-/-}$, we harvested PCs from mice infected for 24 h with HSV-2. The nonadherent cells, which are enriched for NK cells and contained >95% of the NK activity (data not shown), were selected and injected into CCR5$^{-/-}$ mice infected with the virus 2 h previously. Two days later, livers were harvested, and virus titers were determined. As seen in Fig. 5, after 2 days of infection the virus titers in the livers were between 10^3 and 10^4 PFU per g of tissue. Interestingly, if the mice received NK cell activity from C57BL/6 mice, the titers were reduced significantly. In contrast, this was not seen for mice that received NK cell activity from CCR5$^{-/-}$

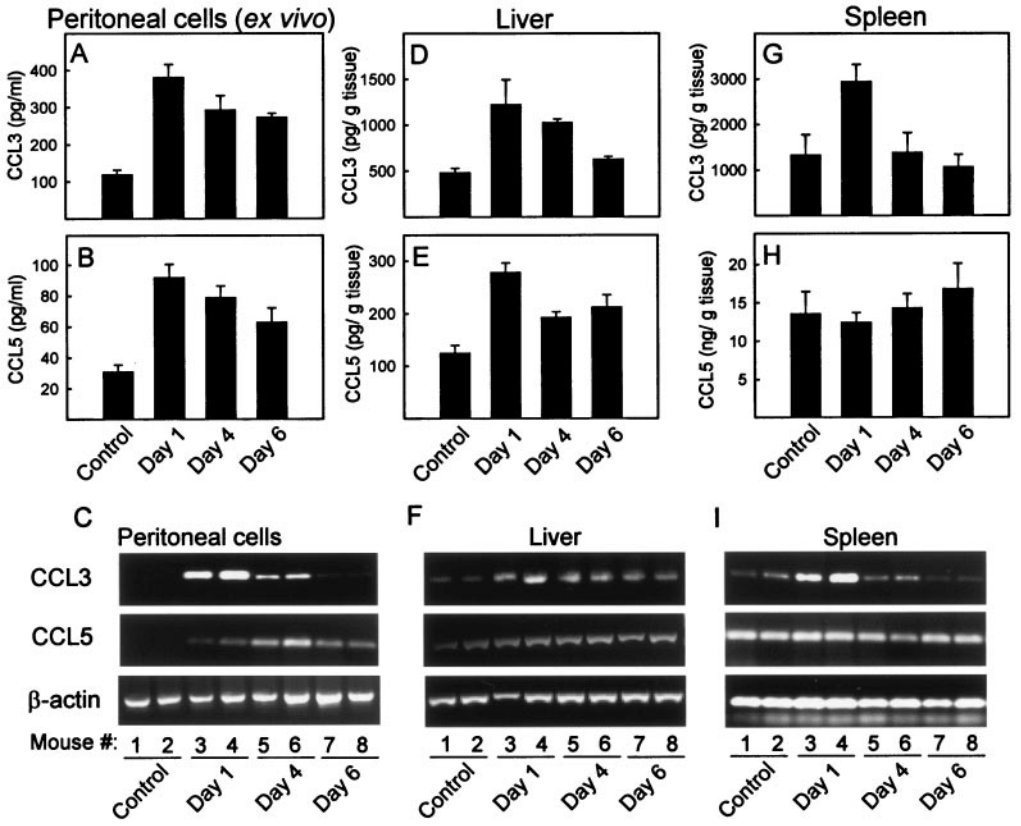

FIG. 2. Expression of CCL3 and CCL5 during a generalized virus infection in 4-week-old mice. Four-week-old female C57BL/6 mice were infected i.p. with 10^6 PFU of HSV-2, and livers, spleens, and PCs were harvested at indicated time points p.i. (A and B) The PCs were cultured for 24 h, and supernatants were harvested for measurement of CCL3 and CCL5 by ELISA. (D, E, G, and H) Livers and spleens were homogenized, and CCL3 and CCL5 were measured in the supernatants. The results are shown as mean ± the SEM ($n = 3$ to 9). (C, F, and I) RNA was isolated from PCs, livers, and spleens from mice treated as indicated. CCL3, CCL5, and β-actin mRNA were detected by RT-PCR.

mice. Therefore, $CCR5^{-/-}$ mice are capable of recruiting NK cells to the site of infection but have a reduced capacity to stimulate cytotoxic activity of these cells and exert antiviral activity, which is not caused by impaired production of IL-12 and IFN-α/β.

Development of spleen enlargement and SC proliferation in response to virus infection. In Fig. 1 we observed that the highest virus titers in the livers of C57BL/6 mice were found on day 4, followed by a dramatic decline between days 4 and 6. In $CCR5^{-/-}$ mice this decrease was not observed to the same extent, which suggested that the adaptive immune response to HSV-2 infection might also be affected by CCR5 deficiency in 4-week-old mice. As the adaptive immune response develops, antigen-specific lymphocytes expand in secondary lymphoid organs. As expected, spleen weight increased in C57BL/6 mice as the infection progressed (Fig. 6A). The same was observed in CCR1-deficient mice. However, in CCR5-deficient mice we did not observe an increase in the weight of the spleen after infection. In these mice we saw a slight increase in the weight of the spleen between days 1 and 4, parallel to the increase seen in the WT and $CCR1^{-/-}$ mice, but the weight did not increase further on day 6, despite the same viral load in the spleen. Likewise, when total numbers of cells in the spleens were determined, we found that the increase in SCs as the immune response developed was much less pronounced in $CCR5^{-/-}$ mice (Fig. 6B).

We wanted to examine whether the spleen enlargement was a result of increased proliferation of SCs. Since splenomegaly developed between days 4 and 6 p.i, we studied ex vivo proliferation of SCs on day 5 after in vivo infection. As seen in Fig. 6C, SCs from infected WT mice showed a proliferative response >2-fold higher than SCs from uninfected mice. In contrast, SCs from CCR5-deficient mice evoked a significantly lower proliferative response after infection. These results suggest that lymphocytes do not expand properly in the spleen in $CCR5^{-/-}$ mice after HSV-2 infection.

Immune responses in the spleen. Since CCR5-deficient mice displayed a strikingly impaired ability to control infection between days 4 and 6 p.i. and also did not develop splenomegaly in response to infection, we wanted to explore the nature of the adaptive immune response to the infection in these mice. First, we looked at IFN-γ production by splenic T cells in response to antigen restimulation. Spleens were harvested from mice infected for 6 days or from control mice, and T cells were isolated and cultured in vitro with irradiated syngeneic SCs as antigen-presenting cells in the presence or absence of heat-inactivated virus. Supernatants were harvested after 72 h of culture, and IFN-γ levels were measured by ELISA. As seen in

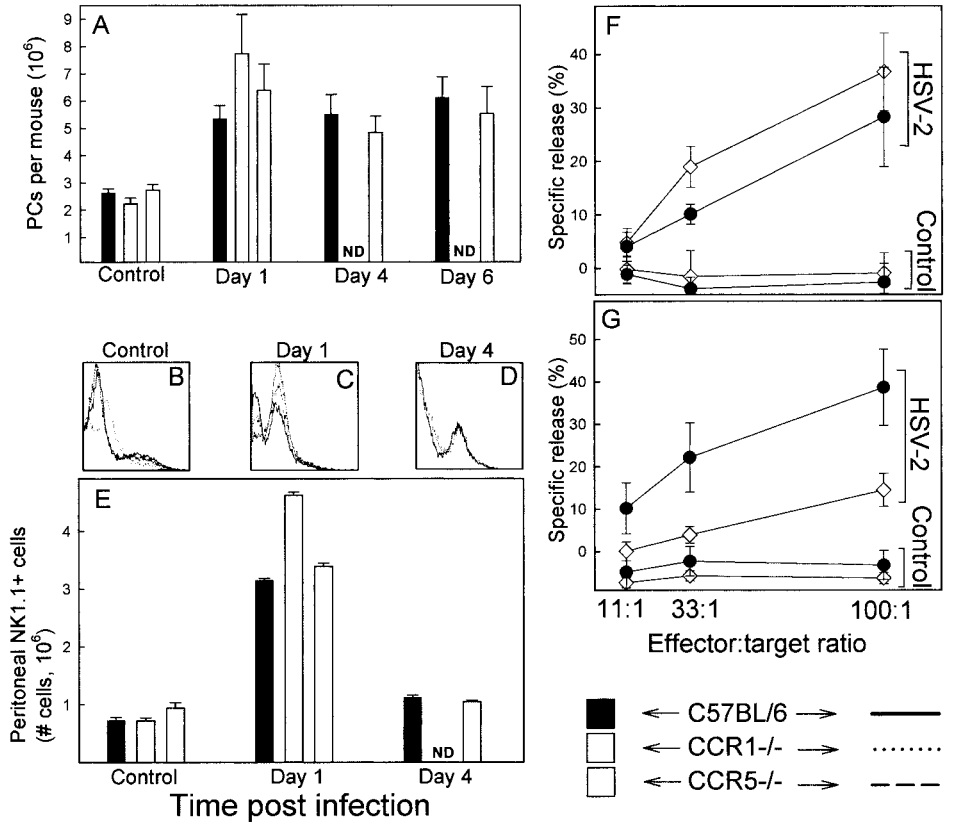

FIG. 3. Accumulation and activation of leukocytes and NK1.1 positive cells in the peritoneal cavity of C57BL/6, CCR1$^{-/-}$, and CCR5$^{-/-}$ mice during HSV-2 infection. (A) PCs from 4-week-old infected and control mice were harvested and counted. The cell numbers are shown as mean ± the SEM. (B to E) Accumulation of NK cells in the peritoneal cavity. NK1.1-positive cells in the PC population were identified by flow cytometry. Representative diagrams are shown in panels B to D, and panel E shows a summary of an analysis of three mice per group. Values are presented as mean ± the SEM. ND, not done. (F and G) NK activity of PCs after HSV-2 infection. PCs were harvested from WT, CCR1$^{-/-}$, and CCR5$^{-/-}$ mice infected for 24 h with HSV-2 and assayed for cytotoxic activity toward YAC-1 cells in different ratios of effector to target cells. The results are shown as mean ± the SEM.

Fig. 7, T cells from uninfected C57BL/6 mice produced no IFN-γ after treatment in vitro with virus antigen. When T cells from infected C57BL/6 mice were examined, restimulation in vitro led to a significant elevation of IFN-γ production compared to cells from uninfected mice. T cells from infected CCR5-deficient mice produced IFN-γ in response to antigen restimulation to an even larger extent than T cells from infected C57BL/6 mice. This indicates that although splenic proliferation of lymphocytes in response to HSV-2 infection is impaired in CCR5$^{-/-}$ mice, antigen-specific T cells do develop in these mice after HSV-2 infection.

To characterize further the splenic response to infection, we examined the populations of T lymphocytes in the spleen. We harvested spleens on days 1, 4, and 6 p.i. and examined the distribution of cells positive for CD3 and CD4 (CD3/CD4) or CD3 and CD8 (CD3/CD8) (Fig. 8). We noted that the spleens of uninfected CCR5-deficient mice contained fewer CD3/CD4-positive (Fig. 8A, E, and I) and CD3/CD8-positive (Fig. 8J, N, and R) cells than the spleens of C57BL/6 mice, although the same proportions of CD3/CD4 positive cells were recovered from the spleens of both mice strains. WT mice were able to respond to HSV infection by increasing both the percentage and the absolute numbers of both CD3/CD4-positive (Fig. 8A to I) and CD3/CD8-positive lymphocytes (Fig. 8J to R), reaching levels significantly different from the knockout mice. At no point during the course of infection did we observe increases in the percentage of CD3/CD4- or CD3/CD8-positive cells in the CCR5-deficient mice, but we did observe a modest increase in the absolute number of CD3/CD8 cells (Fig. 8R).

These data support the findings given above that CCR5$^{-/-}$ mice are defective in lymphocyte expansion during HSV-2 infection and further demonstrate that this defect is seen for both CD4- and CD8-positive T cells. However, this is not reflected in the amount of IFN-γ produced, since T cells from CCR5$^{-/-}$ mice produce IFN-γ to levels at least comparable to what is seen in WT mice after virus rechallenge.

Effector cell migration to the liver. Since the generalized HSV-2 infection in mice develops most strikingly in the liver, and since antiviral activity in this organ was impaired in CCR5-deficient mice, we examined accumulation of T-cell populations in the liver during infection. Livers were harvested from C57BL/6 mice and CCR5-deficient mice on day 5 p.i. and examined for the presence of cells positive for CD3/CD4 or CD3/CD8 (Fig. 9). With respect to CD3/CD4-positive cells, no infection-induced changes were observed, nor were any significant differences between C57BL/6 and CCR5$^{-/-}$ mice appar-

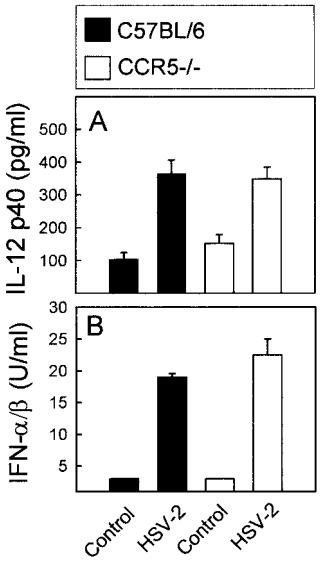

FIG. 4. HSV-induced expression of IL-12 p40 and IFN-α/β in 4-week-old WT and CCR5$^{-/-}$ mice. PCs were harvested from WT and CCR5$^{-/-}$ mice and sat in culture for 24 h in the presence or absence of HSV-2 (3×10^6 PFU/ml). The supernatants were harvested, and the content of IL-12 p40 (A) and IFN-α/β (B) was measured by ELISA and bioassay, respectively. The results are shown as mean ± the SEM.

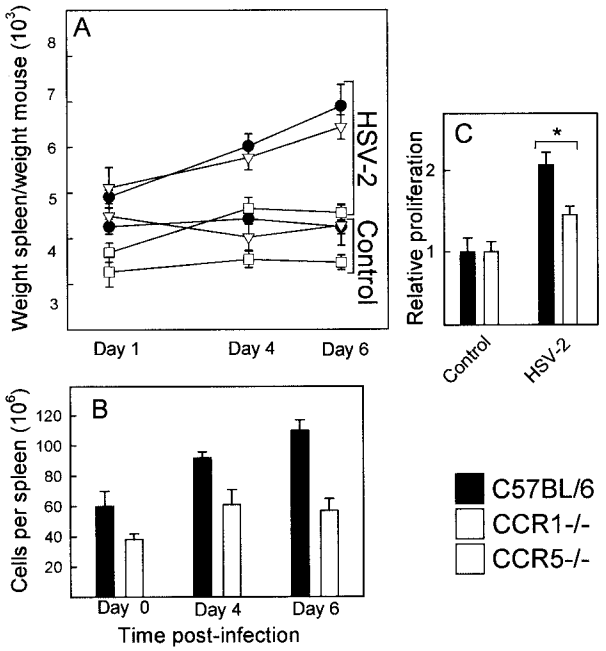

FIG. 6. Expansion of SCs during a generalized virus infection. (A) Spleens were harvested from 4-week-old C57BL/6, CCR1$^{-/-}$, and CCR5 $^{-/-}$ mice at the indicated time points after infection with 10^6 PFU of HSV-2. The weights of the spleens were measured and plotted as a function of time of infection. The results are shown as mean ± the SEM (n = 10 to 50). (B) Spleens were harvested from C57BL/6 and CCR5$^{-/-}$ mice at the indicated times after HSV-2 infection, and SCs were isolated and counted. The results are shown as mean ± the SEM (n = 3 to 6). (C) SCs from C57BL/6 and CCR5$^{-/-}$ mice, either uninfected or infected for 5 days with HSV-2, were harvested and cultured. The proliferation of SCs ex vivo was measured and is shown as the mean ± the SEM (n = 5; P = 0.008).

ent (Fig. 9A to E). In C57BL/6 mice, we observed an increase in the number of CD3/CD8-positive cells on day 5 p.i. compared to uninfected mice. This increase was not seen in the livers from infected CCR5$^{-/-}$ mice (Fig. 9F to J). Thus, in 4-week-old mice lacking CCR5, fewer CD8 T cells are recruited to the liver during an acute generalized infection.

To examine how the impaired proliferative response of splenic lymphocytes from CCR5$^{-/-}$ mice affected the antiviral response, we adoptively transferred SCs from immune C57BL/6 and CCR5-deficient mice to C57BL/6 mice infected 6 h earlier. In the first set of experiments, SCs were given to the recipient mice in the same ratio since they had been recovered from C57BL/6 and CCR5$^{-/-}$ mice (ratio 1.9:1), whereas in the second set, equal numbers of SCs from immune C57BL/6 and CCR5-deficient mice were transferred to the recipient mice.

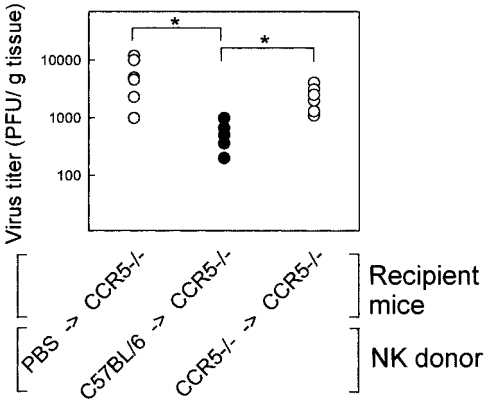

FIG. 5. Adoptive transfer of NK cell activity from C57BL/6 and CCR5$^{-/-}$ mice to HSV-2-infected CCR5$^{-/-}$ mice. PCs were harvested from mice infected for 24 h with HSV-2, and 4×10^6 of the nonadherent cells were injected i.p. into each CCR5$^{-/-}$ mice infected with 10^6 PFU of HSV-2 2 h previously. Two days later the mice were sacrificed, and virus titers in the livers were determined by plaque assay on Vero cells. Statistically significant differences ($P < 0.05$) are marked by asterisks.

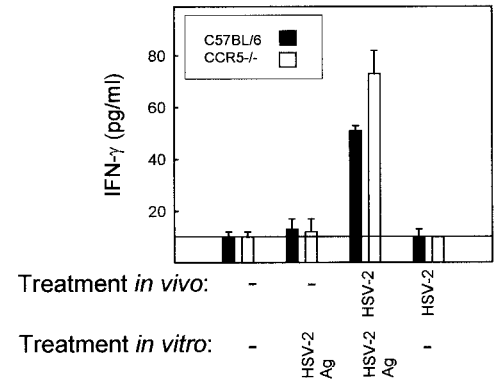

FIG. 7. Production of IFN-γ by T cells from 4-week-old mice infected in vivo and cultured in vitro in the presence or absence of viral restimulation. Splenic T cells were isolated from control and day-6-infected mice and sat in culture for 72 h with irradiated syngeneic SCs in the presence or absence of 3×10^7 PFU of heat-inactivated HSV-2. IFN-γ levels in the supernatants were measured by ELISA. The results are shown as means ± the SEM (n = 5).

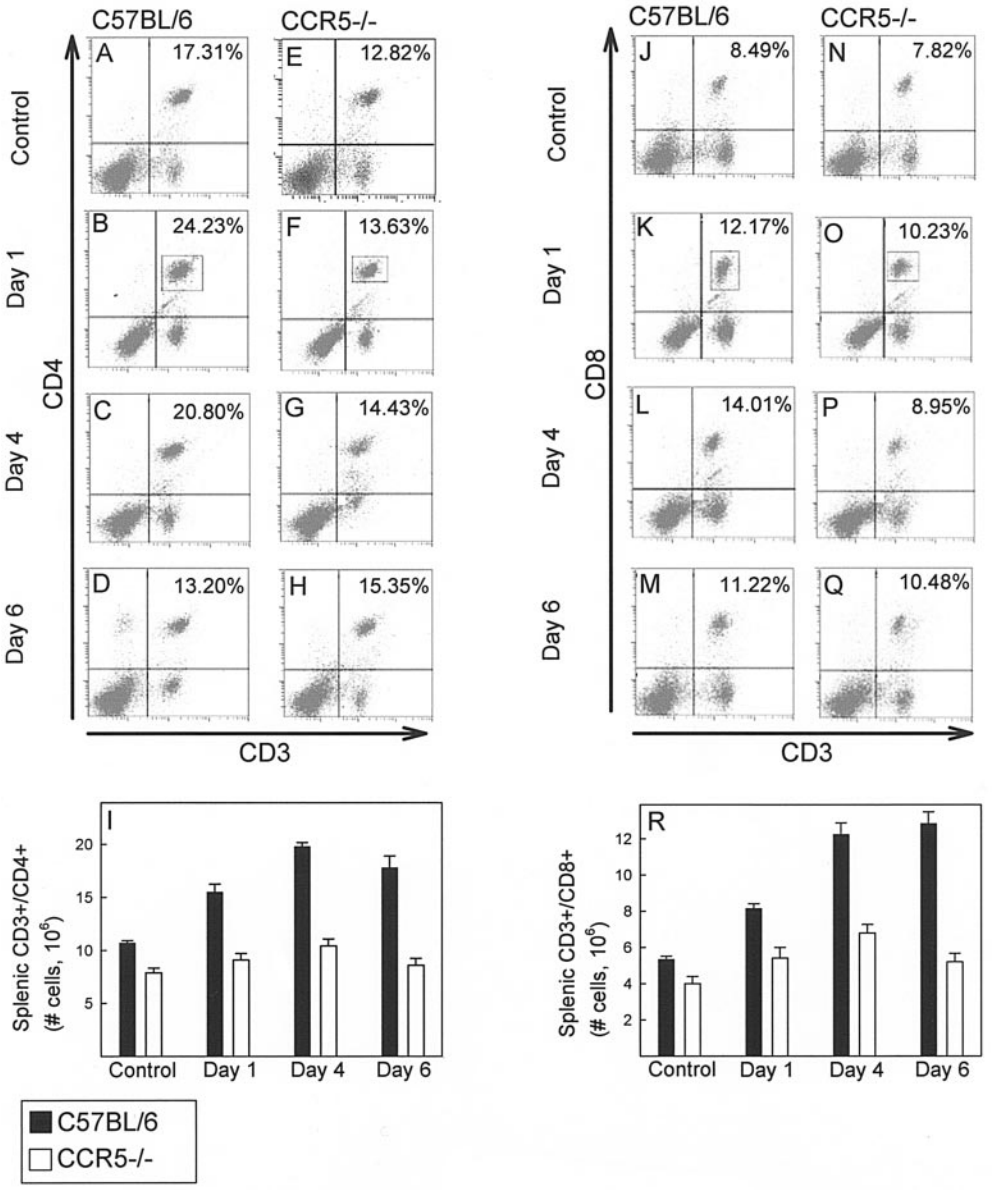

FIG. 8. T-cell subsets in the spleen of C57BL/6 and CCR5$^{-/-}$ mice during HSV-2 infection. CD3/CD4-double-positive (A to I) and CD3/CD8-double-positive (J to R) cells in the spleens of 4-week-old control and virus-infected mice were identified by flow cytometry. Representative dot blots are shown in panels A to H (CD3/CD4) and J to Q (CD3/CD8), whereas panels I and R show summaries of analyses of three mice per group, with the results shown as means ± the SEM.

As seen in Fig. 10, adoptive transfer of immune SCs from C57BL/6 mice to infected mice lowered virus titers in the liver 2 days posttransfer. When SCs isolated from CCR5$^{-/-}$ mice were given to the mice, no significant decrease in virus titers was observed. When the number of SCs transferred reflected the ratio between SCs recovered from immune C57BL/6 versus CCR5$^{-/-}$ mice, the observed differences reached statistical significance.

DISCUSSION

When challenged by infection, the organism mounts an inflammatory response aiming at eliminating the infectious agent. The best-described effector cell populations in defense against viral infections are NK cells and CTLs (CD8$^+$ T cells). NK cells kill virus-infected cells in the early phase of the infection by targeting cells expressing low levels of MHC class I, whereas CD8$^+$ T cells specifically kill cells displaying the appropriate peptides in the context of MHC I via Fas or perforin and granzyme B (35). Consequently, an optimal antiviral defense requires coordinated recruitment of NK cells and antigen-specific T cells to the site of infection, processes in which chemokines play pivotal roles (16, 18, 19). Several studies have investigated the role of chemokines and chemokine receptors during viral infections (20). The results have demonstrated that during some infections specific chemokines or chemokine re-

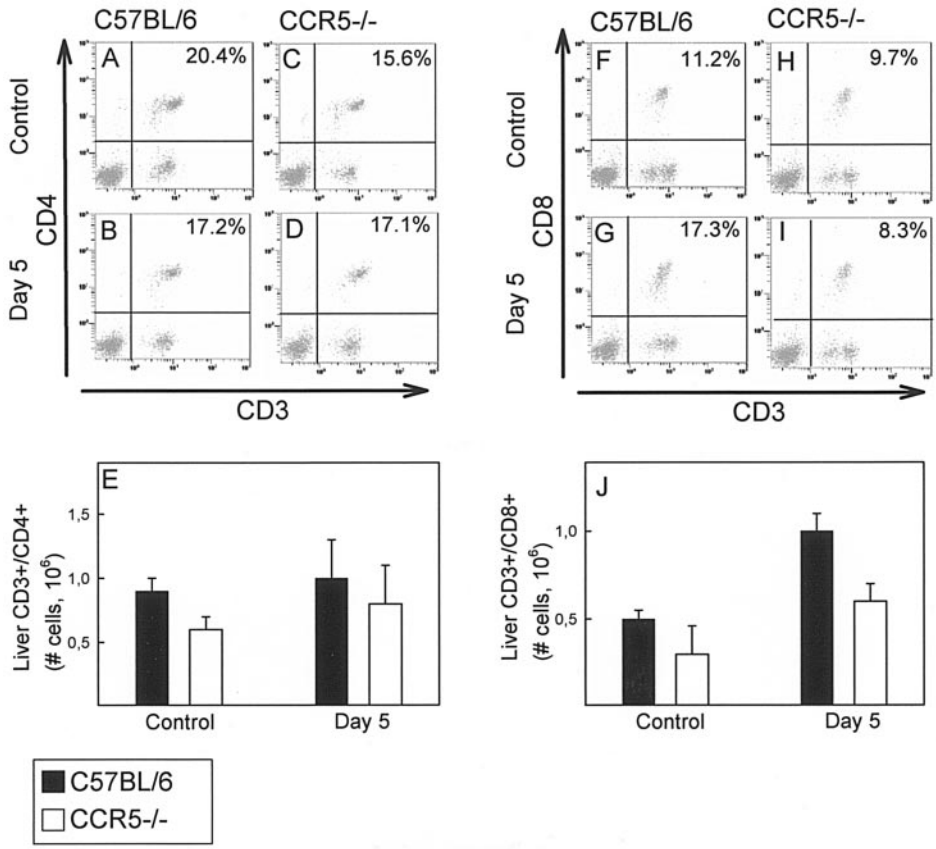

FIG. 9. T-cell subsets in the liver of C57BL/6 and CCR5$^{-/-}$ mice during HSV-2 infection. CD3/CD4-double-positive (A to E) and CD3/CD8-double-positive (F to J) cells in the liver of untreated and virus-infected 4-week-old mice were identified by flow cytometry. Representative dot blots are shown in panels A to D (CD3/CD4) and F to I (CD3/CD8), whereas panels E and J show summaries of analyses of three mice per group, with the results shown as means ± the SEM.

ceptors are required for mounting an efficient antiviral response, (7, 8, 14, 15, 24, 27), whereas in other infections these molecules contribute to an excessive immune response, causing tissue damage (3, 6, 32). In the present study we investigated the role of CCR1 and CCR5 in host defense during an acute generalized virus infection and found that, although this receptor is redundant for viral clearance in adult mice, young mice require CCR5 to mount a strong antiviral cellular innate and adaptive immune response.

Initially, we compared 4- and 8-week-old C57BL/6 mice with CCR1- and CCR5-deficient mice and found that young, but not adult, CCR5$^{-/-}$ mice exhibited impaired clearing of the infection in liver and brain. No significant differences between C57BL/6 and CCR1$^{-/-}$ mice were found with respect to clearance of HSV-2 infections. It has previously been demonstrated that CCR5 is important for clearance of infections by *Cryptococcus neoformans* and *Toxoplasma gondii* (14, 24) but not lymphocytic choriomeningitis virus, MHV, influenza virus, and *Listeria monocytogenes* (5, 22, 26, 36). Our work has thus disclosed an age-dependent role of CCR5 in defense against a virus infection. At least two potential explanations may underlay this finding. First, it is well-known that an age-dependent resistance against infections develops in mice when the animals reach sexual maturity (21, 25), and it is possible that the involvement of multiple mechanisms allows a sufficient antiviral response in adult CCR5$^{-/-}$ mice despite the lack of an antiviral mechanism dependent on this receptor. Second, young mice might harbor a CCR5-dependent antiviral defense mechanism not used in mature animals. For instance, γδ T cells, which do express CCR5 (11), are more abundant early in life than during adulthood (12) and have recently been shown to be required for a protective immune response against an intestinal parasite in 4-week-old mice but not to contribute to defense in 7-week-old mice (25).

NK cells have long been recognized to play an important role in early defense against many viruses, including herpesviruses (2, 23, 28), and Salazar-Mather et al. (27) demonstrated that CCL3-deficient mice had higher levels of NK cells in the blood and spleen, but these cells could not migrate to the liver during a murine CMV infection, hence leading to impaired antiviral defense. When we examined the innate antiviral response at the site of infection, we found a rapid increase in the number of leukocytes and NK1.1-positive cells in the peritoneal cavity with no apparent differences between C57BL/6 mice and any of the knockout mice tested. It should be noted that NK1.1 is not expressed exclusively on NK cells but also on activated CD4- and CD8-positive T cells (30). However, since we examined the cells for NK1.1 expression on day 1 p.i., i.e., before activation of T cells, we believe that the vast majority of NK1.1-positive cells are indeed NK cells. When the cytotoxic

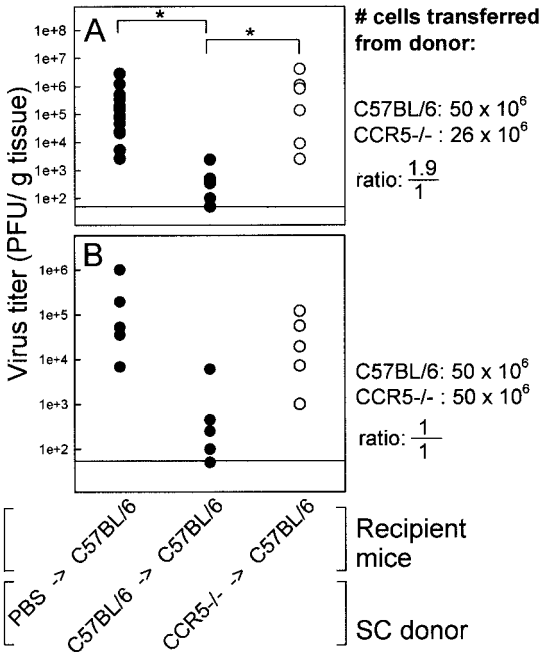

FIG. 10. Adoptive transfer of SCs from C57BL/6 and CCR5$^{-/-}$ mice to HSV-2-infected C57BL/6 mice. Four-week-old C57BL/6 mice were infected i.p. with 10^6 PFU of HSV-2. Six hours later the mice received SCs harvested from 4-week-old C57BL/6 and CCR5$^{-/-}$ mice infected for 6 days with HSV-2. Two days later livers were harvested, and the viral loads in the organs were determined by plaque titration. (A) SCs were given to the recipient mice in the same ratios as they were recovered from the C57BL/6 and CCR5$^{-/-}$ mice in the experiment depicted in Fig. 6 (5×10^7 and 2.6×10^7 cells, respectively). (B) The same number of SCs (5×10^7 cells) were given to the recipient mice irrespective of the number of SCs harvested from the infected C57BL/6 and CCR5$^{-/-}$ mice. The results are shown as dots representing individual mice ($n = 5$ to 13). Statistically significant differences ($P < 0.05$) are marked by asterisks.

activity of the recruited NK cells was examined we found that NK cells harvested from infected CCR5-deficient mice, but not from CCR1-deficient mice, displayed significantly lower activity than NK cells harvested from infected C57BL/6 mice. The underlying mechanism appears to be independent of IL-12 and IFN-α/β production, two well-known activators of NK cell cytotoxic activity (2, 31). The observed reduction in NK cell activity in CCR5$^{-/-}$ mice was associated with lower antiviral capacity in vivo, as assessed by adoptive transfer of NK activity to recipient CCR5$^{-/-}$ mice. In our protocol we transferred nonadherent cells and hence cannot exclude that other cell types, including plasmacytoid DCs with the potential to produce large amounts of IFN-α/β, could also have been delivered to the recipient mice and so contribute to the observed differences between the WT and CCR5$^{-/-}$ mice. However, since C57BL/6 and CCR5$^{-/-}$ mice did not differ with respect to HSV-induced IFN-α/β production we do not think contaminating cell types have affected the result significantly and thus conclude that the differences in viral load after adoptive transfer of NK activity can indeed be ascribed to differences in NK cell activity between C57BL/6 and CCR5$^{-/-}$ mice.

When the animals were examined macroscopically, we observed that, whereas the spleens of WT and CCR1$^{-/-}$ mice increased in size as the infection developed, spleens from CCR5$^{-/-}$ mice retained the same size. No difference between WT and CCR5$^{-/-}$ animals was found when we examined 8-week-old mice (data not shown). In agreement with the differences in spleen size between C57BL/6 and CCR5-deficient mice, we found that SCs harvested from 4-week-old infected CCR5-deficient mice proliferated significantly less ex vivo than cells harvested from infected C57BL/6 mice. When we looked at the T-cell subsets in the spleen, we noted that both CD4 and CD8 T cells were impaired with respect to expansion in response to infection in young CCR5-deficient animals. As for the underlying mechanisms behind this finding, several possibilities exist. First, it is possible that recruitment of immature DCs to the site of infection is impaired in the absence of CCR5. This would lead to reduced antigen presentation to T cells in lymphoid organs and hence the generation of a lower T-cell response. Second, it is possible that antigens are presented to the naive T cells, but proliferative signals are not sufficiently strong in the CCR5-deficient mice. For instance, low production of IL-2 would hamper proliferation. Alternatively, costimulatory molecules on T cells also play essential roles in the regulation of T-cell proliferation (4).

We observed reduced migration of CD8-positive T cells to the liver in the CCR5$^{-/-}$ mice in response to HSV-2, which suggests that the lack of CCR5 cannot be compensated for by other chemokine receptors. A similar defect in CD8$^+$-T-cell trafficking has been observed in CCR5-deficient mice after disseminated *Cryptococcus neoformans* infection (14) or intracranial infection with MHV (10). Adoptive transfer of equal numbers of SCs from immune C57BL/6 and CCR5$^{-/-}$ mice to HSV-2-infected C57BL/6 mice did not recover the antiviral activity of CCR5 SCs, thus suggesting that in addition to the reduced T-cell proliferation in CCR5$^{-/-}$ mice, these cells also display reduced antiviral activity, either due to a defect capacity to migrate to the infected organs or due to reduced function of infiltrating T cells. CCR5 and its ligands have previously been demonstrated to contribute to recruitment of antiviral effector cells to the liver. The CCR5 ligand CCL3 is required for optimal recruitment of NK cells to the liver during CMV infection in mice (27). In humans, ca. 90% of hepatitis B virus (HBV)-specific CD8 T cells that infiltrate the liver of HBV patients are CCR5 positive compared to less than 30% among HBV-specific CD8$^+$ T cells in the blood (29). Moreover, individuals that lack CCR5 exhibit increased susceptibility to hepatitis C virus infection (34).

In summary, we have demonstrated that CCR5, which serves as a coreceptor for HIV and is a potential target for antiviral drugs (33), is required for antiviral defense during a generalized HSV infection in young but not in adult mice. Lack of CCR5 led to impairment of both the innate and adaptive immune response. The present study therefore suggests that treatment of patients with CCR5 antagonists may have negative effects on the immune system in children and immunosuppressed individuals and also highlights the point that modulation of the immune system, as a means of treatment, requires fundamental knowledge of the immune system in different patient groups.

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REFERENCES

1. **Aliberti, J., C. Sousa, M. Schito, S. Hieny, T. Wells, G. B. Huffnagle, and A. Sher**. 2000. CCR5 provides a signal for microbial induced production of IL-12 by CD8 α+ dendritic cells. Nat. Immunol. **1:**83–87.
2. **Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather**. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. **17:**189–220.
3. **Cook, D. N., M. A. Beck, T. M. Coffman, S. L. Kirby, J. F. Sheridan, I. B. Pragnell, and O. Smithies**. 1995. Requirement of MIP-1 α for an inflammatory response to viral infection. Science **269:**1583–1585.
4. **Croft, M**. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat. Rev. Immunol. **3:**609–620.
5. **Dawson, T. C., M. A. Beck, W. A. Kuziel, F. Henderson, and N. Maeda**. 2000. Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. Am. J. Pathol. **156:**1951–1959.
6. **Domachowske, J. B., C. A. Bonville, J. L. Gao, P. M. Murphy, A. J. Easton, and H. F. Rosenberg**. 2000. MIP-1α is produced but it does not control pulmonary inflammation in response to respiratory syncytial virus infection in mice. Cell. Immunol. **206:**1–6.
7. **Domachowske, J. B., C. A. Bonville, J. L. Gao, P. M. Murphy, A. J. Easton, and H. F. Rosenberg**. 2000. The chemokine macrophage-inflammatory protein-1 α and its receptor CCR1 control pulmonary inflammation and antiviral host defense in paramyxovirus infection. J. Immunol. **165:**2677–2682.
8. **Dufour, J. H., M. Dziejman, M. T. Liu, J. H. Leung, T. E. Lane, and A. D. Luster**. 2002. IFN-γ-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T-cell generation and trafficking. J. Immunol. **168:**3195–3204.
9. **Gao, J. L., T. A. Wynn, Y. Chang, E. J. Lee, H. E. Broxmeyer, S. Cooper, H. L. Tiffany, H. Westphal, J. Kwon-Chung, and P. M. Murphy**. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. J. Exp. Med. **185:**1959–1968.
10. **Glass, W. G., M. T. Liu, W. A. Kuziel, and T. E. Lane**. 2001. Reduced macrophage infiltration and demyelination in mice lacking the chemokine receptor CCR5 following infection with a neurotropic coronavirus. Virology **288:**8–17.
11. **Gurney, K. B., O. O. Yang, S. B. Wilson, and C. H. Uittenbogaart**. 2002. TCR $\gamma\delta^+$ and $CD161^+$ thymocytes express HIV-1 in the SCID-hu mouse, potentially contributing to immune dysfunction in HIV infection. J. Immunol. **169:**5338–5346.
12. **Hayday, A. C**. 2000. γδ cells: a right time and a right place for a conserved third way of protection. Annu. Rev. Immunol. **18:**975–1026.
13. **Hellier, S., A. J. Frodsham, B. J. Hennig, P. Klenerman, S. Knapp, P. Ramaley, J. Satsangi, M. Wright, L. Zhang, H. C. Thomas, M. Thursz, and A. V. Hill**. 2003. Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. Hepatology **38:**1468–1476.
14. **Huffnagle, G. B., L. K. McNeil, R. A. McDonald, J. W. Murphy, G. B. Toews, N. Maeda, and W. A. Kuziel**. 1999. Cutting edge: role of C-C chemokine receptor 5 in organ-specific and innate immunity to *Cryptococcus neoformans*. J. Immunol. **163:**4642–4646.
15. **Liu, M. T., D. Armstrong, T. A. Hamilton, and T. E. Lane**. 2001. Expression of Mig (monokine induced by interferon-γ) is important in T lymphocyte recruitment and host defense following viral infection of the central nervous system. J. Immunol. **166:**1790–1795.
16. **Locati, M., and P. M. Murphy**. 1999. Chemokines and chemokine receptors: biology and clinical relevance in inflammation and AIDS. Annu. Rev. Med. **50:**425–440.
17. **Lucotte, G., and G. Mercier**. 1998. Distribution of the CCR5 gene 32-bp deletion in Europe. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. **19:**174–177.
18. **Luster, A. D**. 2002. The role of chemokines in linking innate and adaptive immunity. Curr. Opin. Immunol. **14:**129–135.
19. **Mackay, C. R**. 2001. Chemokines: immunology's high impact factors. Na. Immunol. **2:**95–101.
20. **Melchjorsen, J., L. N. Sorensen, and S. R. Paludan**. 2003. Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. J. Leukoc. Biol. **74:**331–343.
21. **Mogensen, S. C**. 1978. Macrophages and age-dependent resistance to hepatitis induced by herpes simplex virus type 2 in mice. Infect. Immun. **19:**46–50.
22. **Nansen, A., J. P. Christensen, S. S. Andreasen, C. Bartholdy, J. E. Christensen, and A. R. Thomsen**. 2002. The role of CC chemokine receptor 5 in antiviral immunity. Blood **99:**1237–1245.
23. **Orange, J. S., and C. A. Biron**. 1996. Characterization of early IL-12, IFN-α/β, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. J. Immunol. **156:**4746–4756.
24. **Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya**. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. Nat. Immunol. **4:**161–167.
25. **Ramsburg, E., R. Tigelaar, J. Craft, and A. Hayday**. 2003. Age-dependent requirement for γδ T cells in the primary but not secondary protective immune response against an intestinal parasite. J. Exp. Med. **198:**1403–1414.
26. **Roizman, B., and P. R. J. Roane**. 1964. Multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology **22:**262–269.
27. **Salazar-Mather, T. P., T. A. Hamilton, and C. A. Biron**. 2000. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. J. Clin. Investig. **105:**985–993.
28. **Salazar-Mather, T. P., J. S. Orange, and C. A. Biron**. 1998. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1α (MIP-1α)-dependent pathways. J. Exp. Med. **187:**1–14.
29. **Shimada, N., K. Yamamoto, M. J. Kuroda, R. Terada, T. Hakoda, H. Shimomura, H. Hata, E. Nakayama, and Y. Shiratori**. 2003. HBcAg-specific CD8 T cells play an important role in virus suppression, and acute flare-up is associated with the expansion of activated memory T cells. J. Clin. Immunol. **23:**223–232.
30. **Slifka, M. K., R. R. Pagarigan, and J. L. Whitton**. 2000. NK markers are expressed on a high percentage of virus-specific $CD8^+$ and $CD4^+$ T cells. J. Immunol. **164:**2009–2015.
31. **Trinchieri, G**. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. **3:**133–146.
32. **Tumpey, T. M., H. Cheng, D. N. Cook, O. Smithies, J. E. Oakes, and R. N. Lausch**. 1998. Absence of macrophage inflammatory protein-1α prevents the development of blinding herpes stromal keratitis. J. Virol. **72:**3705–3710.
33. **Veazey, R. S., P. J. Klasse, T. J. Ketas, J. D. Reeves, M. Piatak, Jr., K. Kunstman, S. E. Kuhmann, P. A. Marx, J. D. Lifson, J. Dufour, M. Mefford, I. Pandrea, S. M. Wolinsky, R. W. Doms, J. A. DeMartino, S. J. Siciliano, K. Lyons, M. S. Springer, and J. P. Moore**. 2003. Use of a small molecule CCR5 inhibitor in macaques to treat simian immunodeficiency virus infection or prevent simian-human immunodeficiency virus infection. J. Exp. Med. **198:**1551–1562.
34. **Woitas, R. P., G. Ahlenstiel, A. Iwan, J. K. Rockstroh, H. H. Brackmann, B. Kupfer, B. Matz, R. Offergeld, T. Sauerbruch, and U. Spengler**. 2002. Frequency of the HIV-protective CC chemokine receptor 5-Δ32/Δ32 genotype is increased in hepatitis C. Gastroenterology **122:**1721–1728.
35. **Wong, P., and E. G. Pamer**. 2003. CD8 T-cell responses to infectious pathogens. Annu. Rev. Immunol. **21:**29–70.
36. **Zhong, M. X., W. A. Kuziel, E. G. Pamer, and N. V. Serbina**. 2004. Chemokine receptor 5 is dispensable for innate and adaptive immune responses to *Listeria monocytogenes* infection. Infect. Immun. **72:**1057–1064.